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RESPIRATORY TULAREMIA: COMPARISON OF SELECTED ROUTES OF VACCINA--ETC(U)  
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Fischer 344 rats were given attenuated live vaccine strain (LVS) of Francisella tularensis by small particle aerosol, intranasal (i.n.) instillation, or by intraperitoneal (i.p.), intramuscular (i.m.), or subcutaneous (s.c.) injection. All of the vaccinated rats developed subclinical infection by 3 days after exposure, which cleared by day 28. Temporal patterns and concentrations of the LVS organisms within the hosts were dependent upon the route of vaccination. Pathologic alterations were limited to minimal lung lesions in aerosol

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vaccinated rats and mild splenitis in i.p. vaccinated rats. Agglutinins to LVS were detected in the serum of each vaccinated animal and in the bronchoalveolar wash fluids of 66% of the aerosol-vaccinated rats. The agglutinins appeared to be exclusively of the immunoglobulin M class. Regardless of route of vaccine administration, all vaccinated rats survived an aerosol challenge of  $5.3 \log_{10}$  cells of virulent F. tularensis while all non-vaccinated rats died. Systemic infection did not occur in the vaccinated rats. Pulmonary infection, however, was not prevented in the vaccinated rats following aerosol challenge, but proliferation of the virulent F. tularensis organisms in the lungs was significantly lower ( $P < 0.01$ , analysis of variance) than that which occurred in the control animals. These studies demonstrate the utility of the inbred Fischer 344 rat as a model host for further investigations of F. tularensis infection and its associated immune response.

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Fort Detrick, Frederick, Maryland 21701

Running title: VACCINATION AND IMMUNITY TO RESPIRATORY TULAREMIA

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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## ABSTRACT

Fischer 344 rats were given attenuated live vaccine strain (LVS) of Francisella tularensis by small particle aerosol, intranasal (i.n.) instillation, or by intraperitoneal (i.p.), intramuscular (i.m.), or subcutaneous (s.c.) injection. All of the vaccinated rats developed subclinical infection by 3 days after exposure, which cleared by day 28. Temporal patterns and concentrations of the LVS organisms within the hosts were dependent upon the route of vaccination. Pathologic alterations were limited to minimal lung lesions in aerosol-vaccinated rats and mild splenitis in i.p. vaccinated rats. Agglutinins to LVS were detected in the serum of each vaccinated animal and in the bronchoalveolar wash fluids of 66% of the aerosol-vaccinated rats. The agglutinins appeared to be exclusively of the immunoglobulin M class. Regardless of route of vaccine administration, all vaccinated rats survived an aerosol challenge of  $5.3 \log_{10}$  cells of virulent F. tularensis while all nonvaccinated rats died. Systemic infection did not occur in the vaccinated rats. Pulmonary infection, however, was not prevented in the vaccinated rats following aerosol challenge, but proliferation of the virulent F. tularensis organisms in the lungs was significantly lower ( $P \leq 0.01$  analysis of variance) than that which occurred in the control animals. These studies demonstrate the utility of the inbred Fischer 344 rat as a model host for further investigations of F. tularensis infection and its associated immune response.

The stimulation of immunity in the lungs by respiratory pathogens has been considered an important factor in host resistance (19). Recent approaches to research on this issue have involved depositing an immunogen in the respiratory tract by intranasal instillation or aerosol inhalation (5, 6, 9). In our laboratory, different methods of vaccination, including inhalation of aerosols of microbial antigens are under investigation as a means for inducing immunity against respiratory diseases, e.g., respiratory tularemia. Several different strains of rats have been used with varying results in previous studies on the pathogenesis and resistance to tularemia (2, 10, 13, 17, 20). Preliminary dose-response evaluations in our laboratory showed the inbred Fischer 344 rat to be consistently susceptible to infection with virulent F. tularensis, and that immunity could be conferred to this rat strain with the attenuated live vaccine strain (LVS) of F. tularensis. Initial studies, therefore, emphasized characterization of the pathogenesis of F. tularensis infection in Fischer 344 rats with the ultimate goal being to elucidate the relative importance of the component arms of the immune response in providing protection against lethal respiratory tularemia.

This report presents the response of Fischer 344 rats to infection caused by the administration of the LVS of F. tularensis by small particle aerosol, or by the intranasal, intraperitoneal, intramuscular, or subcutaneous routes. Also evaluated was the protection afforded these LVS-vaccinated rats after challenging them with aerosols of virulent F. tularensis, SCHU S4.

## MATERIALS AND METHODS

Animals. Inbred, male Fischer 344 rats {CDF<sup>R</sup> (F-344)/Cr1 BR} were procured from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Rats (200-240 g) were housed four to six per cage and given water and commercial feed pellets (Ralston-Purina, St. Louis, Mo.) ad libitum. After challenge with virulent F. tularensis, SCHU S4, the rats were maintained in total containment biological safety cabinets (7).

F. tularensis cultures. Both attenuated, live vaccine and virulent SCHU S4 strains of F. tularensis were cultured in modified casein partial-hydrolysate (MCPH) liquid medium and stored at -60°C as previously described (3). Frozen cultures were thawed just before use. Viable cell concentrations were estimated by plating 10-fold serial dilutions on glucose-cysteine-blood-agar (GCBA) plates. Typical colonies were counted after incubation for 48 to 72 h at 37°C.

Vaccination procedures. For intranasal (i.n.) vaccination, a 20- $\mu$ l volume of LVS suspension was instilled by micropipette (Eppendorf) into the anterior nares of rats lightly anesthetized with halothane. Preliminary studies using carbon black as an indicator system showed that the 20  $\mu$ l were retained entirely in the upper respiratory tract (unpublished data). Other groups of anesthetized rats were vaccinated by intraperitoneal (i.p.), intramuscular (i.m.), or subcutaneous (s.c.) injection with 0.5 ml LVS suspension. Aerosol vaccination was performed by exposing unanesthetized rats in a modified Henderson aerosol apparatus (16) for 10 min to small particle aerosols (SPA) of LVS generated from a Collison spray device (12). The mass median diameter of the aerosol particles was 2  $\mu$ m. With this procedure, the aerosol was deposited predominantly in the lung. Control animals were sham-vaccinated by exposing them similarly to aerosols of sterile MCPH broth. LVS aerosol concentrations were determined by collecting aerosol samples in all-glass impingers (8) containing 20 ml of sterile



gelatin-saline and plating suitable dilutions on GCBA. The presented aerosol dose for the exposed animals was calculated on the basis of LVS concentration per liter of aerosol x minute respiratory volume of a 250 g rat (0.13 liters/min) x duration of animal exposure in minutes (8).

Challenge procedures. Aerosol challenge of the vaccinated and control rats with virulent *F. tularensis*, SCHU S4, and determination of aerosol challenge dose were conducted in the same manner as described for aerosol vaccination. The only exception being that all manipulations were performed in total containment biological safety cabinets (8).

Assay procedures. Groups of rats were killed periodically after vaccination or challenge. Lungs, spleens, and cervical and mediastinal lymph nodes were aseptically excised. Sections of these tissues were processed for histological examination and the balance of each was homogenized in gelatin saline diluent. Appropriate dilutions of the homogenates were streaked on duplicate GCBA plates for determination of viable organism concentrations. The plates were incubated for 48 to 72 h at 37°C, and typical colonies were enumerated. Numbers of organisms in blood and in bronchoalveolar wash (BAW) fluids were estimated by plating 0.1 ml of undiluted samples in duplicate on GCBA. The BAW fluids were obtained by washing the air passages of the lungs with 3 ml of sterile phosphate-buffered saline, pH 7.2, instilled via the trachea. Gentle aspiration of the saline two to three times in the lungs resulted in obtaining 1.5 to 2 ml fluid with no gross signs of blood.

Antibody assays. Agglutinating antibody titers for sera and BAW fluids were measured using microtiter procedures (11). The BAW fluids were concentrated to 0.5 ml by filtration through 25,000-dalton ultra filters (Millipore Corp., Bedford, Mass.) and maintained at -20°C until assayed for antibody content. The concentrations of selected classes of immunoglobulins (IgG, IgA, IgM) of sera

and BAW fluids were estimated by radial immunodiffusion procedures (14), using specific anti-rat Ig prepared in rabbits or goats (Miles Laboratories, Inc., Elkhart, Ind.).

Rat strain susceptibility evaluation. Preliminary studies were conducted to compare the susceptibility of inbred Fischer 344 rats procured from two commercial sources (Charles River Breeding Laboratories and M. A. Bioproducts, Walkersville, Md.) and that of an outbred Sprague-Dawley rat strain (Taconic Farms, Germantown, N.Y.) to aerosol challenge and to i.p. challenge with virulent F. tularensis, SCHU S4.

Experimental protocol. Within the three experiments reported here groups of 50 to 70 Fischer 344 rats were vaccinated with LVS by aerosol exposure to SPA, by i.n. instillation, or by i.p., i.m., and s.c. injections. Control animals in each experiment were exposed to sterile broth aerosols in the same manner as the LVS aerosol-vaccinated group. At 1, 3, 7, 10, 14, and 42 days after vaccination, a selected number of rats were killed by exposure to lethal doses of halothane. Sera and BAW fluids were obtained for serological assay, and tissues were cultured for LVS organisms. The remaining control and vaccinated rats were challenged with virulent F. tularensis aerosols 42 days after initial exposure to broth medium or vaccination. At 2, 3, and 14 days after aerosol challenge, 6 to 7 rats from each group were killed, tissue samples were obtained, assayed for concentrations of challenge organisms, and examined for histologic evidence of tularemia.

Statistical analysis. One way analyses of variances were computed on the logarithms of organism concentrations in selected tissues from vaccinated rats, from tissues of challenged rats, and for the serum IgM levels in vaccinated rats.

## RESULTS

Mortality response of rat strains to tularemia infection. The mortality of Fischer 344 inbred rats and an outbred Sprague-Dawley rat strain was compared following aerosol challenge with virulent F. tularensis, SCHU S4 (Table 1). The effect of aerosolization on organism virulence also was determined by comparing the mortality of rats injected i.p. with F. tularensis collected prior to and after aerosolization. The Fischer rats, from both sources, were highly susceptible to lethal infection with F. tularensis. More than 90% of these rats died 5 to 13 days after aerosol exposure to  $5 \log_{10}$  cells of F. tularensis and within 2 to 7 days after i.p. infection with either the pre- or postaerosolization of organisms. The Sprague-Dawley rats were fully resistant to aerosol challenge and a combined 56% of the rats died following both types of i.p. challenge with F. tularensis. Based on these results, the Fischer 344 rat was selected for use in subsequent studies on LVS vaccination and protective immunity generated against respiratory tularemia.

LVS population dynamics as a function of method of vaccination. The distribution and persistence of LVS in selected tissues of rats vaccinated by different routes was determined over 28 days. The LVS assay data were recorded for lungs, spleen, and lymph nodes through day 14 only because LVS was rarely recovered thereafter (Fig. 1). None of the tissues of sham-vaccinated rats contained LVS; these negative data are not shown. In addition, data have not been presented for rats vaccinated by either i.m. or s.c. injections because, for these animals, the patterns of LVS distribution, concentration and clearance from tissues and appearance of agglutinin titers were so similar to the patterns described for the i.n. vaccinated rats.

All of the rats vaccinated by aerosol exposure yielded LVS organisms from the lungs (Fig. 1). Significantly higher concentrations of organisms

(5.0 to 7.0  $\log_{10}$ ) were present in the lungs of these rats on each assay day, except day 1, as compared to the rats vaccinated by the i.n. or i.p. routes ( $P \leq 0.01$ , analysis of variance). Surprisingly, on day 1 after exposure, LVS organisms were cultured from the lungs of 89% of the rats vaccinated by i.p. injection, and at concentrations not statistically different from those determined for the aerosol-vaccinated rats. LVS organisms were not isolated from the lungs of i.n. vaccinated rats until day 3, with the maximum concentration not exceeding 3.0  $\log_{10}$ . For all three groups of rats, there was a progressive diminution of viable tularensis organisms in the lungs through day 14. No organisms were recovered from any rat by day 28.

Spleens of the i.p. vaccinated rats contained more LVS organisms ( $P \leq 0.01$ , analysis of variance) on days 1 and 3 than did those of the SPA and i.n. vaccinated rats. From day 7 through 14, concentrations of LVS organisms in the spleen were approximately equal in all rats, and ranged from 2 to 4  $\log_{10}$ . As with the lungs, spleens had been cleared of LVS by 28 days.

In general, 3 to 4  $\log_{10}$  of LVS organisms were recovered from a pool of cervical and mediastinal lymph nodes of each rat regardless of route of vaccination. The lymph nodes also were cleared of LVS organisms by day 28.

Blood and BAW fluids were sampled for the presence of LVS organisms and serum and BAW fluids were assayed for LVS agglutinin titers. A transient and low-level bacteremia occasionally was observed among the aerosol and i.n. vaccinated rats. Low levels of LVS organisms ( $< 100$  colony forming units/ml of blood), however, were recovered from all of the i.p. vaccinated rats through day 3. The BAW fluids from all SPA vaccinated rats yielded LVS organisms for 14 days at concentrations that did not exceed 3  $\log_{10}$ . The LVS also was recovered from the BAW fluids from one-third of the i.p. vaccinated rats between days 3 through 10. Viable LVS bacteria were not detected in the BAW fluids of any of the i.n. vaccinated group.

Serum and BAW fluids were assayed for LVS agglutinin titers (Table 2). For all vaccinated groups, serum titers were evident by day 7. Generally the titers peaked by day 10 and subsided to their lowest levels (1:200) by day 42. The highest serum titers were produced by i.p. vaccination. Low-titered BAW agglutinins were detected for about two-thirds of the SPA vaccinated rats through day 10. Serologically positive BAW fluids were detected only on day 7 for one-third of the i.p. vaccinated rats and for one-third of the i.n. vaccinated animals only on day 10.

Changes in concentrations of the different classes of immunoglobulins were detected only for IgM (Fig. 2). Serum IgM levels in both aerosol and i.p. vaccinated rats were significantly higher than those of the nonvaccinated control rats from day 7 through day 14 ( $P \leq 0.05$ , analysis of variance). The serum IgM concentration for the i.n. vaccinated rats was increased significantly only at day 14. IgM also was detected in the BAW fluids of some of the SPA vaccinated rats on days 7 and 10 and in one i.p. vaccinated rat on day 7. The increase in IgM levels in both serum and BAW fluids paralleled the increase and abatement of the LVS agglutinin titers in these fluids (Table 2).

Response of LVS vaccinated animals to aerosol challenge. At 42 days after LVS vaccination, the sham-vaccinated control rats and the five groups of vaccinated rats were challenged with a mean aerosol dose of  $5.3 \log_{10}$  cells of virulent *F. tularensis*, SCHU S4. The data summarized in Fig. 3, however, are for the control, aerosol, i.n., and i.p. vaccinated groups of rats only. All of the control rats appeared ill three days after aerosol challenge. By day 7 after challenge, concentrations of challenge organisms ranged from  $7 \log_{10}$  in the lungs to about  $5 \log_{10}$  in the spleen and lymph nodes of the control rats despite the presence of a high serum antibody titer (mean = 1:1178). Of these rats, 94% died between 6 and 14 days, leaving only two for assay. In the three

groups of vaccinated rats, the overall mean serum agglutinin titer was 1:248 (range 160 to 285).

Although none of these vaccinated rats died or showed signs of clinical illness for a period of 90 days after aerosol challenge, pulmonary infection was not prevented (Fig. 3). After day 2, the peak concentrations of tularensis organisms in the lungs of the vaccinated animals were 2 to 3  $\log_{10}$  lower than those in the control rats ( $P \leq 0.01$ , analysis of variance). Histologically, pulmonary lesions associated with F. tularensis infection were not detected. The vaccinated rats therefore, apparently were protected against respiratory tularensis even though significant numbers of virulent tularensis organisms were present in the lungs. In contrast, the typical pyogranulomatous lesions of tularemia were observed in the lungs of the control rats.

Infection was not evidenced in the spleens of rats in each of the vaccinated groups at 2 days after challenge. Furthermore, organisms were isolated from only one rat in each of these groups and only on day 7 postchallenge. The challenge organism was isolated, however, from one-third of the control rats on day 2 postchallenge and from all the remaining control animals on day 7 and day 14. Lesions typical of tularemia were observed only in the spleens of the nonvaccinated control animals. Infection of the cervical and mediastinal lymph nodes was present in all vaccinated groups of rats as well as in the control animals up to day 14. Concentrations of challenge organisms in the lymph nodes and the percentage of vaccinated rats with infected nodes approximated that obtained for the nonvaccinated control rats.

Bacteremia was demonstrable in all of the control rats sampled from 2 to 7 days after challenge. Concentrations of F. tularensis approximated 3  $\log_{10}$ /ml of blood. None of the vaccinated rats yielded a positive blood culture, except for one i.p. vaccinated rat from which small numbers of organisms were recovered

on day 7. The serum agglutinin titers of the vaccinated rats were no higher 48 h following aerosol challenge than they were just prior to challenge. BAW fluid agglutinin titers were not detected after challenge at any time.

All of the rats vaccinated by i.m. or s.c. injections also survived the aerosol challenge with no overt signs of illness. All other response parameters of the i.m. and s.c. rats to challenge closely resembled those observed for the i.n. rats. The data for the i.m. and s.c. rats, therefore, were not presented in this report.

## DISCUSSION

Vaccination of inbred Fischer 344 rats with the LVS F. tularensis via i.p., i.m. or s.c. injection, i.n. instillation, or aerosol inhalation resulted in temporal patterns and concentrations of the LVS organism with the vaccinated host that were dependent on the route of administration. Regardless of the method of vaccination, none of the rats became febrile or exhibited overt signs of illness even though 3 to 7 log<sub>10</sub> of LVS organisms were recovered from lungs, spleens and lymph nodes through 14 days. Histologically the minimal lung lesions in the aerosol-vaccinated rats and the mild splenitis in the i.p. vaccinated rats were analogous to previously described pathologic alterations in Fischer 344 rats infected by i.p. inoculation with LVS (13). Thus, the subclinical infection induced by the attenuated vaccine strain of F. tularensis was not altered by the route of administration. A consistent bacteremia, indicative of the hematogenous transport of the LVS vaccine, occurred only in the i.p. vaccinated rats between days 1 and 3. Bacteremia rarely was observed in rats vaccinated by aerosol inhalation or by i.n. instillation suggesting that with these methods of vaccination, systemic spread of the vaccine strain with these two methods of vaccination occurs early by way of the lymphatic system. The LVS organisms were cleared from all tissues of the vaccinated rats after 14 days regardless of vaccination method.

Serum LVS agglutinins were induced readily by all methods of vaccination with the highest titers being obtained by the i.p. route. Antibodies were detected 3 days after vaccination which reached peak levels usually by day 10. The increase in antibody production coincided with a general decrease in concentration of LVS in the tissues and with the frequency of host infection. By day 12, antibody titers leveled off at 1:126 to 1:240. At 18 months after either aerosol- or i.m. vaccination, serum titers of 1:20 to 1:80 still were



detectable (unpublished data). The immunoglobulin class associated with the agglutinin activity was IgM which corroborates the previous observations in humans (1); adult male volunteers given a percutaneous vaccination with  $8.0 \log_{10}$  of LVS cells developed demonstrable antibody within 7 days that was predominantly IgM. The LVS agglutinins detected in the BAW fluids of the aerosol-vaccinated rats also were comprised of IgM. Previous evidence indicated that IgM was not a normal constituent of respiratory fluids (15), even though a variable number of IgM producing cells reside within the respiratory tract parenchyma (18). Under appropriate stimuli, such as inhalation of an aerosolized antigen, it could be that such cells would synthesize IgM antibodies. It is possible also that transudation of serum IgM across respiratory mucous membranes may have occurred, as significant levels of IgM existed simultaneously in both serum and BAW fluids of the aerosol-vaccinated rats. The data available, however, do not substantiate transudation of serum IgM into the bronchi and related spaces. Of all the rats tested after i.p. injection or i.n. instillation of LVS, only one i.p. vaccinated rat exhibited detectable IgM in BAW fluids even though IgM was demonstrable in the serum of all these animals. Thus, the presence of surface IgM within the lungs most likely results from direct stimulation of antibody producing cells. Definition of the explicit source of the surface IgM and the role of these antibodies in respiratory tularemia requires further investigation.

All rats vaccinated with F. tularensis LVS regardless of route (aerosol, i.n., i.p., i.m., s.c.) were protected against a lethal challenge with the virulent F. tularensis, SCHU S4. None of the vaccinated rats, each possessing low level antibody titers evidenced overt clinical signs, developed detectable bacteremia or died. By contrast, all of the nonvaccinated control rats exhibited definite signs of illness and became bacteremic despite the development of high titers (mean 1:1178) of serum agglutinin. Of these animals, 95% died after

challenge. Accordingly, it would appear that pre-existing serum antibodies may help to prevent systemic infection in challenged rats. In Gram-negative bacterial infection IgM has the propensity, in conjunction with complement, to coat and lyse bacteria. Complexing of the pre-existing circulating IgM antibodies with the bacterial antigen may explain the lack of a bacteremia, development of minimal pathology, and the absence of clinical infection in vaccinated rats while lethal infections occurred in the challenged control rats despite the presence of concurrently produced high titers of serum antibody. Antigen-antibody complexes also could explain the absence of an anamnestic reaction in the vaccinated rats after challenge. A similar lack of rise in serum antibody titers in vaccinated volunteers following aerosol challenge has been reported (1). These cumulative data imply that pre-existing humoral antibodies may play an important complimentary role against clinical tularemia in concert with cell-mediated immunity, which has been reported to be the prime immune response against tularemia (4). These data also lead to the conclusion that immunity to lethal respiratory tularemia can be as effectively induced by either aerosol inhalation or i.n. instillation of LVS antigen as by the injection of LVS by the i.p., i.v. or s.c. route.

The inbred Fischer 344, therefore, provides an excellent model for further investigations of *F. tularensis* infection and its associated immune responses. Comparative studies using nonimmune, immune, immunosuppressed, and immunopotentialized rats can be designed to evaluate the relative importance of the effector arms of immunity to respiratory tularemia, specifically, and to respiratory diseases, in general.

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TABLE 1. Mortality of rats exposed to F. tularensis, SCHU S4

Mode of challenge	Challenge dose ( $\log_{10}$ )	% Mortality (days to death)		
		MA-F <sup>a</sup> (n = 12)	CR-F <sup>b</sup> (n = 8)	Tac-SD <sup>c</sup> (n = 8)
SPA <sup>d</sup>	5.04	92 (3-12)	100 (5-13)	0
i.p. <sup>e</sup> (pre-aerosol)	5.5	100 (2-5)	100 (2-4)	50 (4-5)
i.p. (post-aerosol)	5.11	100 (3)	88 (3-7)	63 (3-9)

<sup>a</sup>MA-F: Microbiological Associates - Fischer 344

<sup>b</sup>CR-F: Charles River - Fischer 344

<sup>c</sup>Tac-SD: Taconic Farms - Sprague-Dawley

<sup>d</sup>SPA: Small particle aerosol

<sup>e</sup>i.p.: Intraperitoneal injection

TABLE 2. Serum and BAW agglutinin titers in LVS vaccinated rats

Vaccination route <sup>a</sup>	% of rats with titer by days (mean titer <sup>b</sup> )				
	Fluid	7	10	14	42
SPA	Serum	100 ( 352)	100 (1478)	100 (628)	100 (126)
	BAW	67 ( 24)	67 ( 20)	0	0
i.n.	Serum	73 ( 278)	78 ( 452)	100 (666)	80 (226)
	BAW	0	33 ( 10)	0	0
i.p.	Serum	100 (1574)	100 (2030)	100 (878)	100 (240)
	BAW	33 ( 20)	0	0	0

<sup>a</sup>Geometric mean  $\log_{10}$  vaccination dose over all routes =  $5.1 \pm 0.59$ .

<sup>b</sup>Reciprocal of positive animals.

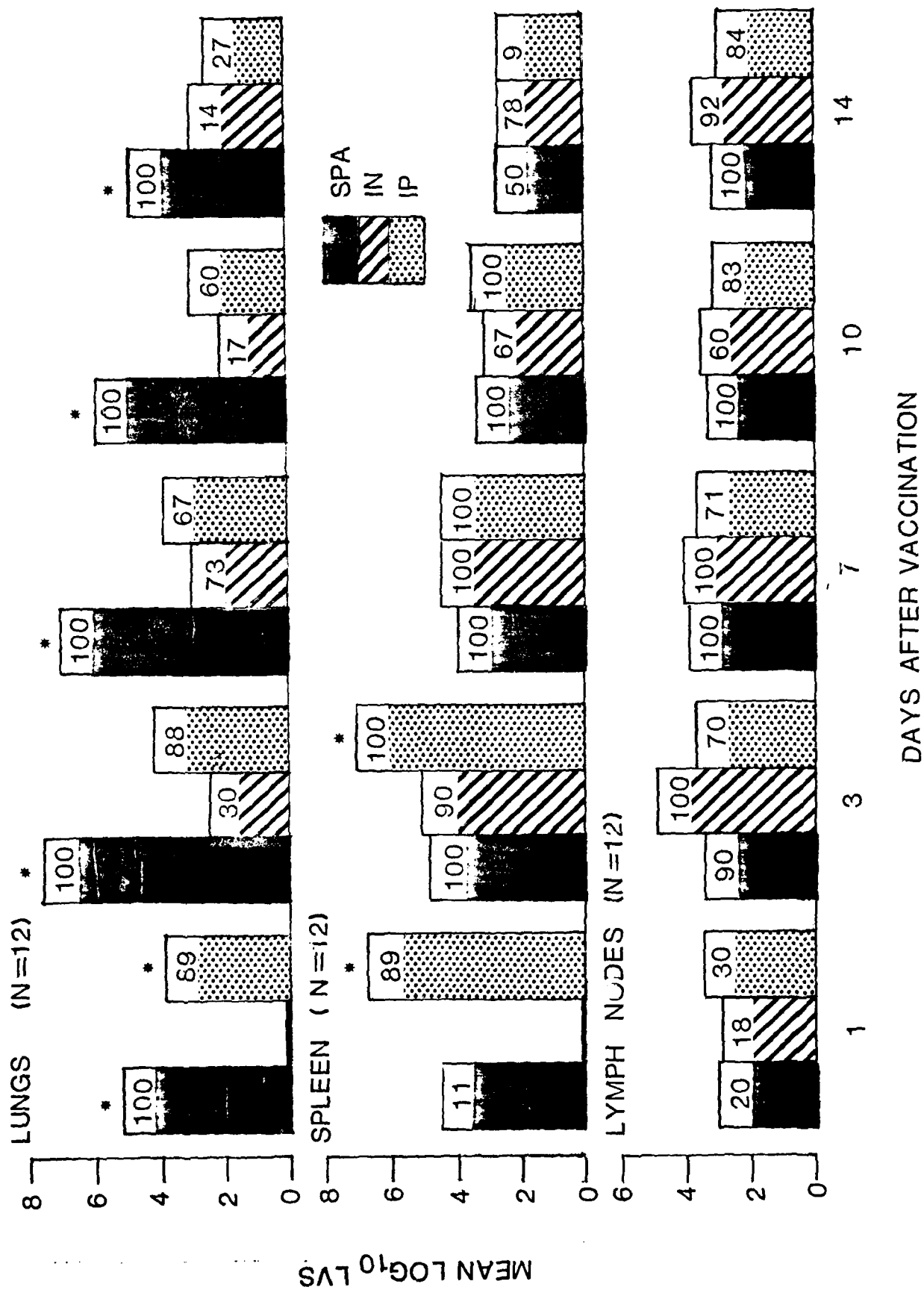


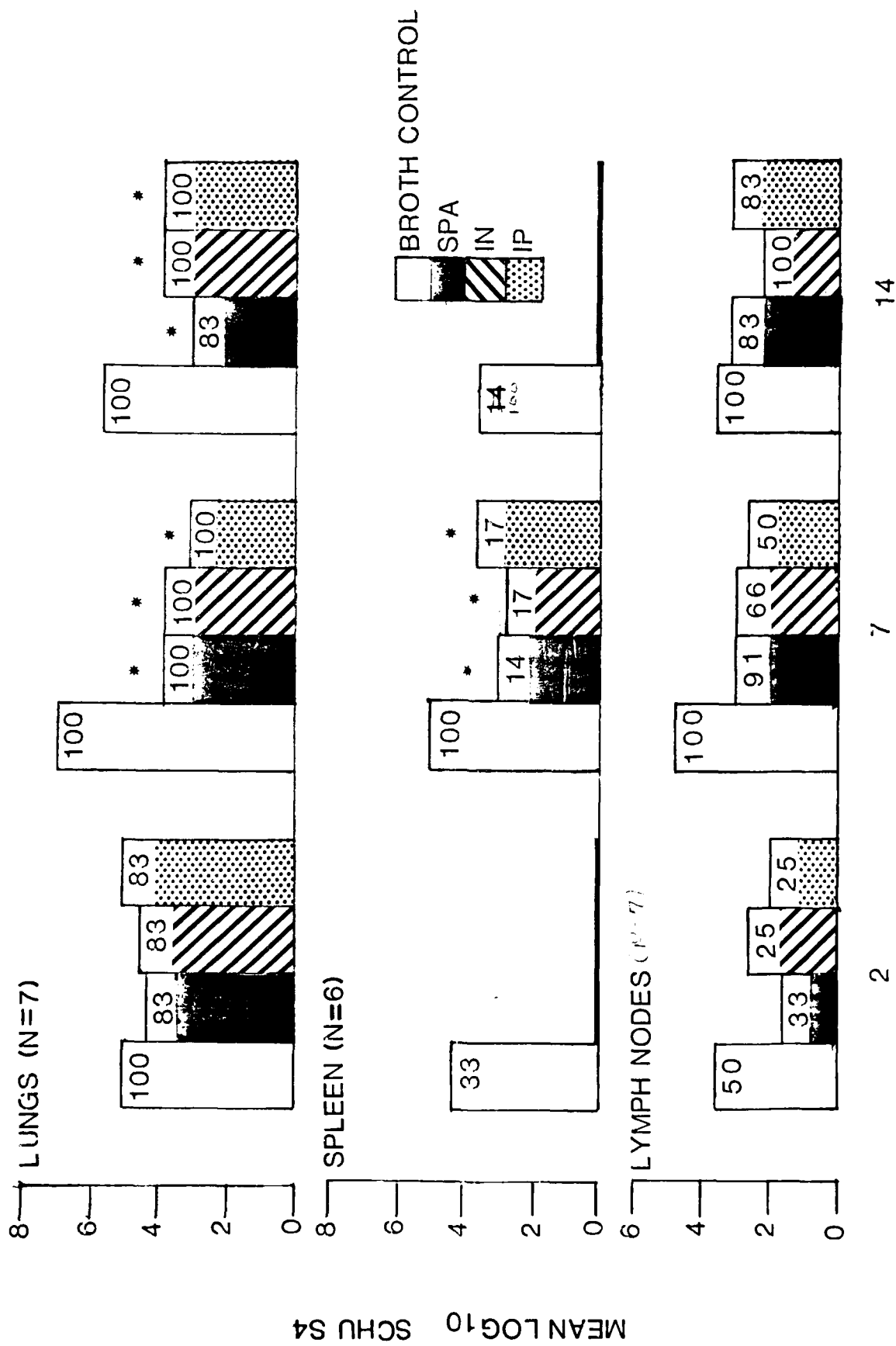
## FIGURE LEGENDS

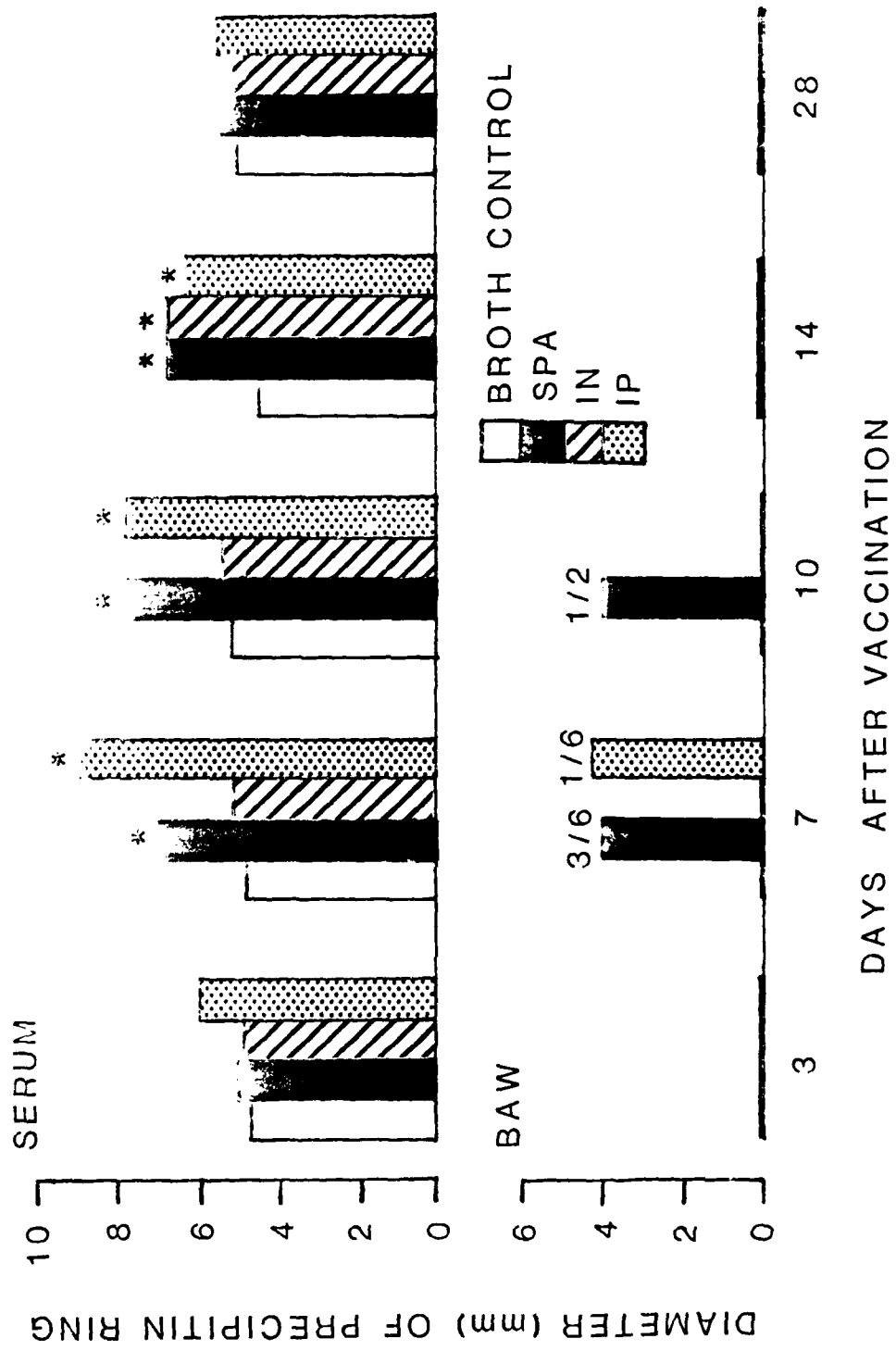
Fig. 1. Mean number ( $\log_{10}$ ) of LVS organisms in lungs, spleens, and lymph nodes of F-344 rats at various days after vaccination either by SP aerosols, i.n., or i.p. routes (geometric mean  $\log_{10}$  dose over all routes equals  $5.1 \pm 0.59$ ). The number at the top of each bar indicates the percent of rats from which LVS was recovered. The asterisk above a bar indicates a LVS concentration significantly higher ( $P \leq 0.01$ , analysis of variance) than other in the same group.

Fig. 2. Relative levels of IgM in serum and BAW fluids as measured by radial immunodiffusion and based on the diameter (mm) of the precipitin ring. The mean diameter of the precipitin ring was calculated on the basis of three replicate samples per rat (n = 6). The asterisk (\*) above a bar for the serum samples indicates an IgM concentration significantly higher ( $P \leq 0.05$ , analysis of variance) than the broth control. The numbers over the bars for the BAW samples indicate the proportion of the rats with IgM concentrations increased over baseline values.

Fig. 3. Mean  $\log_{10}$  challenge organisms in lungs, spleens, and lymph nodes after exposure to an aerosol containing  $10^{5.3}$  virulent *F. tularensis*. Number at the top of each bar indicates the percent of rats from which tularensis organisms were recovered. The asterisk above a bar indicates an organism concentration significantly lower ( $P \leq 0.01$ , analysis of variance) than the broth control.







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